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Avian influenza A virus PB2 promotes interferon type I inducing properties of a swine strain in porcine dendritic cells

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ABSTRACT

The 2009 influenza A virus (IAV) pandemic resulted from reassortment of avian, human and swine strains probably in pigs. To elucidate the role of viral genes in host adaptation regarding innate immune responses, we focussed on the effect of genes from an avian H5N1 and a porcine H1N1 IAV on infectivity and activation of porcine GM-CSF-induced dendritic cells (DC). The highest interferon type I responses were achieved by the porcine virus reassortant containing the avian polymerase gene PB2. This finding was not due to differential tropism since all viruses infected DC equally. All viruses equally induced MHC class II, but porcine H1N1 expressing the avian viral PB2 induced more prominent nuclear NF-κB translocation compared to its parent IAV. The enhanced activation of DC may be detrimental or beneficial. An over-stimulation of innate responses could result in either pronounced tissue damage or increased resistance against IAV reassortants carrying avian PB2.

Introduction

Influenza A viruses (IAV) can infect a broad variety of hosts, ranging from birds to humans, but the natural reservoir for IAV is wild water fowl in which all 16 subtypes of hemagglutinin and all 9 subtypes of neuraminidase (NA) known so far are circulating. Transmission between the different hosts is restricted due to different receptor specificities of the HA. Avian IAV, for example, binds to N-acetylneuraminic acid (NeuAc) α-2.3 linked galactose (Gal) predominantly, whereas human IAV preferentially recognize NeuAcα2,6-Gal receptors (Matrosovich et al., 2004, 2007; van Riel et al., 2006). Newly emerging IAV subtypes can become a threat for the human population, as no immunity against them would be present. So far, direct transmissions of HPAIV from avian hosts to humans were very rare events. However, pigs can be infected by both human and avian IAV. In case of simultaneous infections, reassortant viruses could emerge and then transmit to humans (Horimoto and Kawaoka, 2005; Kawaoka et al., 1989; Ma et al., 2008; Scholtissek et al., 1978). Such a scenario very likely initiated the 2009 influenza pandemic in which an IAV, composed of genes of avian, porcine and human virus origin, was transmitted from pigs to humans. In contrast to the H5N1 HPIAV, this virus efficiently spread from human to human world-wide and therefore caused a pandemic (Michaelis et al., 2009).

Beside the HA, the viral polymerase has been demonstrated to mediate host specificity. The IAV polymerase complex consists of three proteins, the PA, PB1 and PB2. A number of adaptive mutations in these genes which enhance the replication of avian IAV in mammalian cells, were identified. For example, polymerase complexes originating from avian IAV show cold-sensitivity in mammalian cells, due to the higher temperature at the main replication site in birds (gastrointestinal tract) compared with humans (upper respiratory tract). This temperature sensitivity can be reversed by mutation of glutamic acid to lysine at position 627 of PB2, leading to increased replication efficiency in humans (Massin et al., 2001).

Those changes in HA or polymerase genes reflect the adaptation of IAV to host factors indicating that host factors shape the survival of the virus in the new species. In light of the pivotal role of the innate immune system in suppressing early virus replication and in initiation of adaptive immune responses, the interaction of the virus with elements of the innate immune system as the first line of defense is of utmost importance. Dendritic cells (DC) are central for the interplay between the innate and adaptive immune systems, and thus, their interaction with viruses is crucial for further clearance. During IAV infection, viral antigen can be detected in lung DCs. Furthermore, in vitro infection of DCs by IAV has been reported to induce their maturation and activation resulting in antiviral innate and adaptive immune responses. IAV can induce antiviral mediators, mainly IFN type I limiting early viral replication, the secretion of inflammatory cytokines like IL-6 and TNF-α responsible for the recruitment of DC and other inflammatory cells to the site of infection, and the upregulation of MHCII, CD40, CD83, CD86 and CCR7 necessary for the migration of DC

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to draining lymph nodes and ultimately for the priming of naïve T and B cells (Bender et al., 1998; Fonteneau et al., 2003; Perrone et al., 2008; Thitithanyanont et al., 2007). On the other hand, based on their potency to sense and respond to IAV, DCs could contribute to the "cytokine storm" believed to be responsible for some aspects observed during severe IAV infection in humans (de Jong et al., 2006).

So far, many studies scrutinizing the interaction of DCs and IAV have been conducted in mice, which are not a natural host. Therefore, those data may not fully be applicable a natural mammalian hosts such pigs. Considering the possible role of pigs as intermediate host for transmission of avian IAV to man, we studied the ability of one avian and one porcine IAVto infect and activate porcine GM-CSF derived DCs. To elucidate the role of the single gene segments, we generated recombinant reassortant viruses. We found, that amongst those reassortants, the PB2 determines the activation of GM-CSF derived DCs in terms of nuclear NF-κB translocation and IFN type I secretion.

Results

Avian polymerase changes the IFN-inducing properties of porcine IAV in GM-CSF derived DCs

In view of the central functions of IFN type I acting both as direct antiviral factor and a cytokine strongly promoting adaptive effector T and B lymphocyte responses, we screened various reassortants for their ability to induce IFN type I in GM-CSF-derived porcine DC (from now on termed "DC"). We used an avian highly pathogenic (HP) H5N1 (A/chicken/Yamaguchi/7/04) and a porcine H1N1 (A/swine/Belzig/2/01) strain as well as reassortants thereof generated by reverse genetics. We exchanged the HA, NS1 and polymerase genes to elucidate their impact on innate immune responses either through regulating cell susceptibility to infection, replication efficiency or direct interference with the antiviral response. To test antiviral activity of infected DCs, we analyzed the supernatants of infected cells for IFN type I activity by bioassay. Only the swine H1N1 containing the avian set of polymerases (Sw-Y_PA/PB1/PB2), but neither of the parental viruses nor the H5N1 strain containing the swine IAV polymerase (Y-Sw_PA/PB1/PB2) induced detectable IFN bioactivity at 5 h post infection (p.i.) (Fig. 1A). When supernatants harvested at 20 h p.i. were analyzed all viruses induced detectable bioactivity but the levels were overall lower, and the swine IAV with the avian polymerases was the most stimulatory (Fig. 1B).

This difference in IFN type I inducing properties of the tested viruses was also confirmed on the level of cellular mRNA. 5 h p.i. we detected a 5000 fold induction of IFN-β mRNA in DCs infected with the swine IAV containing the avian polymerase (Sw-Y_PA/PB1/PB2; MOI of 10 IU/ml) compared to control allantoic fluid (CAF). The other IAV tested caused only minor IFN-β mRNA induction (Fig. 1C). Compared to this, the IFN-α mRNA induction 5 h p.i. was low (Fig. 1D). We also blocked the bioactivity of IFN-α using a neutralizing polyclonal serum and found no reduction in bioactivity with supernatants harvested at 5 h p.i. These data

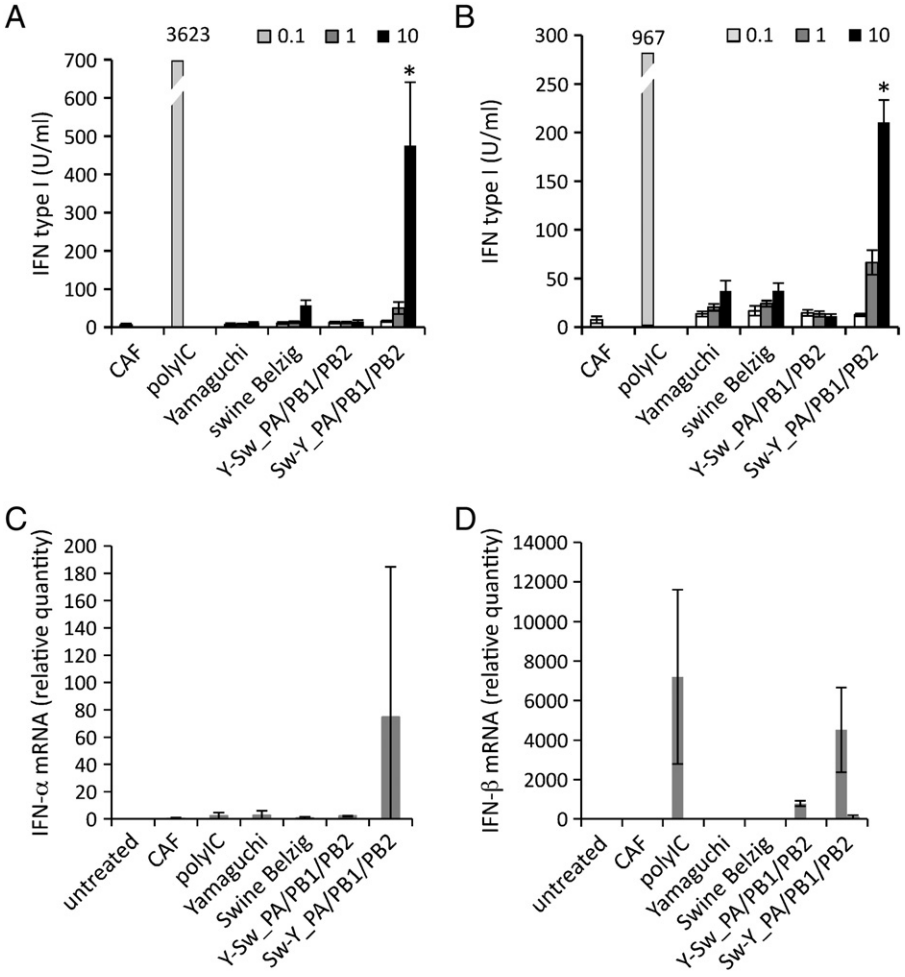


Fig. 1. Avian polymerase changes the IFN-inducing properties of porcine IAV in DCs. GM-CSF DCs were infected at three different MOIs (0.1, 1, 10 IU/cell). 5 h (A) and 20 h (B) p.i. supernatants were harvested and tested for IFN type I activity by bioassay. The mean of triplicates cultures with standard errors from one experiment representative for three independent experiments is shown. Only Y-Sw_PA/PB1/PB2 at an MOI of 10 IU/cell induced a significantly higher level of IFN type I when compared to the CAF control (*, $p<0.05$). In C and D, the relative quantities of INF-β and IFN-α mRNA measured by real-time RT-PCR at 5 h p.i. with an MOI of 10 IU/cell are shown. The IFN mRNA levels relative to the 18S mRNA levels were calculated. The mean of triplicates with standard errors of triplicate cultures are shown.

indicating that the IFN type I response was mostly IFN-β were confirmed by IFN-α ELISA in which no or IFN levels below 5U/ml were detected (data not shown).

Exchanging HA or NS1 between the avian H5N1 and the swine H1N1 did not result in a virus able to induce IFN type I at 5 h p.i. (data not shown). Considering these results the investigations of the present study focussed only on the role of the polymerases in infection and activation of porcine DC.

DCs are infected and activated by both avian and porcine IAV

To investigate the susceptibility of DCs to IAV infection we analyzed nucleoprotein (NP) expression at 5 and 20 h p.i. We found that all IAV tested, independent of the origin of the polymerase genes, were equally able to infect DCs and start replication in terms of NP expression, and that high levels of NP were detected at 20 h p.i. (Fig. 2A). At 5 h p.i., the percentage of NP^+ DCs was MOI-dependent starting at around 5–10%

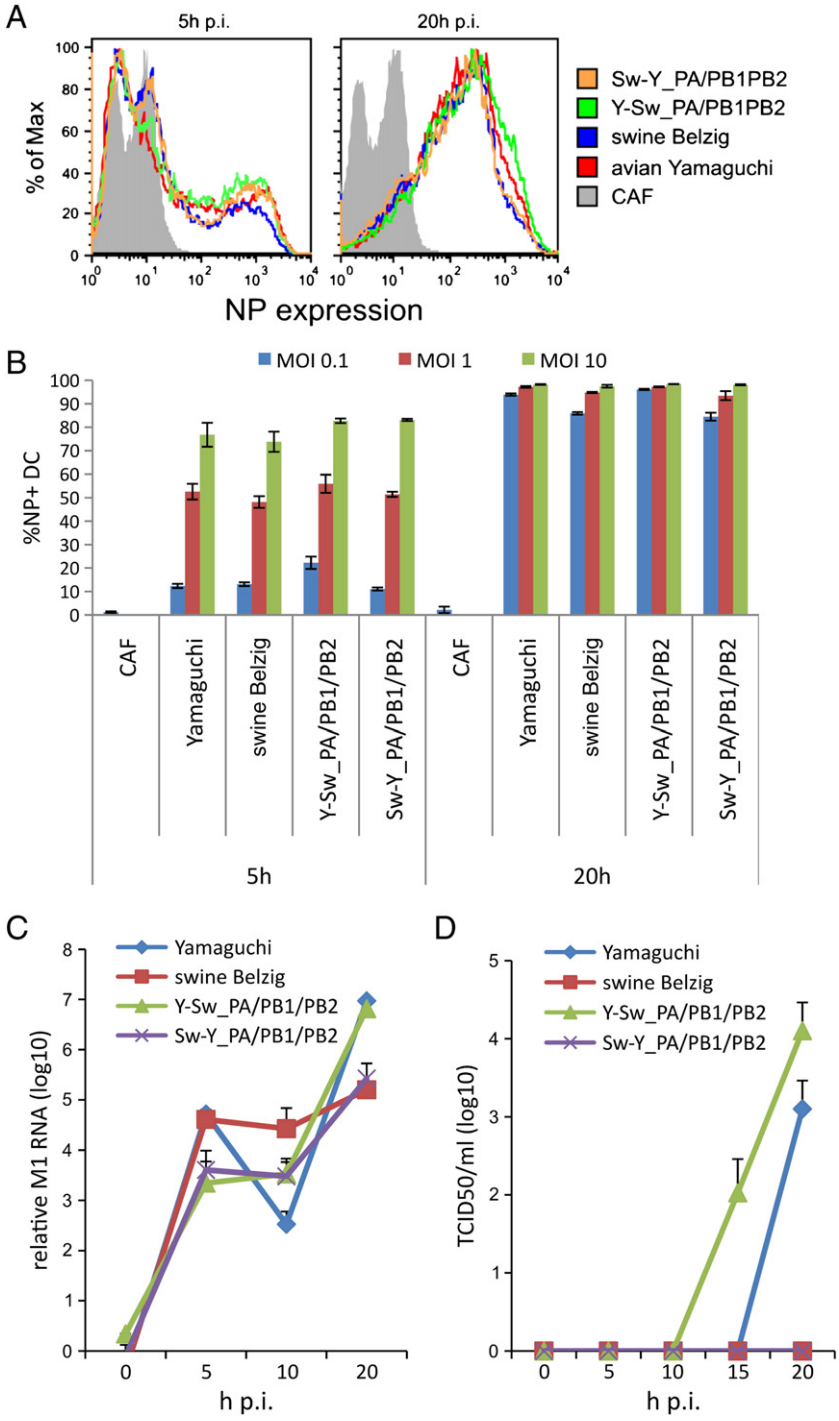


Fig. 2. Infection and replication of IAV in DCs. GM-CSF derived DCs were infected at three different MOIs (0.1, 1, 10 IU/cell). In (A), a representative FCM histogram profile of NP expression at 5 and 20 h p.i. with an MOI of 0.1 IU/cell is shown. In (B), the mean and standard errors of triplicate cultures of the percentage of NP^+ DCs infected with MOI's of 0.1, 1 and 10 IU/cell and analyzed at 5 and 20 h p.i. is shown. The data is representative of three independent experiments. NP^+ DC obtained after infection with Y-Sw_PA/PB1/PB2 and Sw-Y_PA/PB1/PB2 did not differ significantly when compared to the parental viruses, avian Yamaguchi and swine Belzig, respectively ($p > 0.05$). In (C), the viral M1 RNA levels of DC infected with an MOI of 0.1 IU/cell were quantified by real-time RT-PCR and shown relative to the 18S RNA. Mean and standard errors of triplicate cultures were calculated. In (D), supernatants obtained at various time points were tested for progeny virus by titration on MDCK cells. Mean and standard errors of triplicate cultures are shown.

with MOI of 0.1 IU/cell, and reaching values above 50% with an MOI of 10 IU/cell (Fig. 2B). At 20 h p.i. the percentage of NP^+ DCs reached 80% or more for all viruses and MOIs tested (Fig. 2B).

The increase of NP^+ DC from around 10% at 5 h p.i. to almost 100% at 20 h p.i. indicated replication of IAV in DC. This was confirmed by the observation that infection with UV-inactivated virus did not result in NP^+ cells (data not shown) and by the increase of viral RNA determined by real-time RT-PCR (Fig. 2C). We did not observe a higher rate of viral RNA with the swine virus containing the avian polymerase. We also tested the supernatants of infected DC cultures for newly released virus. Only infection with viruses containing the HA of the Yamaguchi virus led to a detectable increase in viral titres 24 h p.i., although the titres remained low (320 $TCID_{50}$/ml for the Yamaguchi and 1264 $TCID_{50}$/ml for the Y-Sw_PA/PB1/PB2 virus, Fig. 2D). This indicated that the polybasic cleavage site promotes a more efficient multicycle replication in GM-CSF derived DCs. Addition of trypsin to enhance replication of viruses with a monobasic HA cleavage site did not help to confirm the role of HA cleavage due to toxicity of the protease in DC cultures. Nevertheless, we also tested another pair of avian H5N1 influenza viruses with monobasic and polybasic cleavage sites, composed of a low pathogenic H5N1 vaccine strain (A/vac-1/Hokkaido/04) and the same virus with the Yamaguchi HA (Moulin et al., 2011). Again only with the highly pathogenic biotype an increase in viral titres in the supernatant was observed (data not shown).

Enhanced MHC class II expression on DC is induced by both avian and porcine IAV independent of the polymerase origin

In order to further characterize the activation of DCs during IAV infection we also examined MHC class II (MHCII) upregulation at 24 h p.i. All viruses induced a potent (relative to polyIC) increase in MHCII surface expression with MOIs of 1 and 10 IU/cell (Fig. 3), differing significantly from CAF ($p<0.01$; calculated using *t*-test). For viruses containing the Yamaguchi HA (Yamaguchi and Y-Sw_PA/PB1/PB2) the staining intensity did not further increase with an MOI of 10 IU/cell. This was most probably due to cytopathogenic and/or toxic effects observed in these cultures. Altogether, the similar capacity to induced MHCII contrasted with the differential ability to induce IFN type I, and indicated that the avian polymerase gene in the porcine IAV context specifically influences IFN type I responses, and that the IAV-induced MHCII upregulation in DC was at least partially IFN-independent.

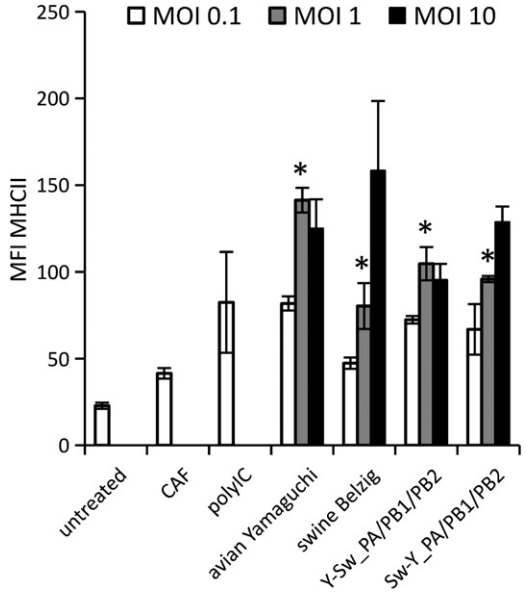


Fig. 3. Induction of phenotypic maturation of DC by IAV. GM-CSF derived DCs were infected at three different MOIs (0.1, 1, 10 IU/cell) and stained for MHCII at 24 h p.i.. Mean fluorescence intensity is shown for triplicates of a representative experiment out of three. For MOI of 1 and 10 IU/cell all viruses induced significantly higher expression of MHCII when compared to the CAF control (*, $p<0.01$).

Avian PB2 suffices to change IFN inducing properties of porcine IAV in DCs

To further analyze the role of the avian polymerases on the IFN inducing properties of the swine IAV, we rescued recombinant swine IAV with either PA (Sw-Y_PA), PB1 (Sw-Y_PB1) or PB2 (Sw-Y_PB2) of the avian Yamaguchi virus. The results demonstrated that exchanging PB2 was sufficient to induce IFN type I in infected DCs (Figs. 4A and B). Only in one out of three experiments we also found some IFN type I in the supernatants of DCs infected with the Sw-Y_PB1 and Sw-Y_PA (Fig. 4C). However, these IFN levels were significantly lower (50–60 U/ml) compared to Sw-Y_PA/PB1/PB2 (201 U/ml; $p<0.05$; calculated by *t* test), whereas the levels found with Sw-Y_PB2 did not differ significantly from the reassortant virus with all three polymerase genes (Sw-Y_PA/PB1/PB2, $p>0.05$; calculated by *t* test). Also the supernatants harvested at 24 h p.i. had the highest levels of IFN type I when the DC were infected with swine viruses containing PB2 of avian IAV origin (data not shown).

Individual avian polymerases do not influence porcine DCs infection and MHCII expression

To determine the impact of infection on the above results, NP expression was analyzed after 5 h of culture as described above. No statistically significant difference was observed when the original swine virus was compared to reassortant viruses containing one of the avian polymerases (Fig. 5A; $p>0.05$; calculated by *t* test).

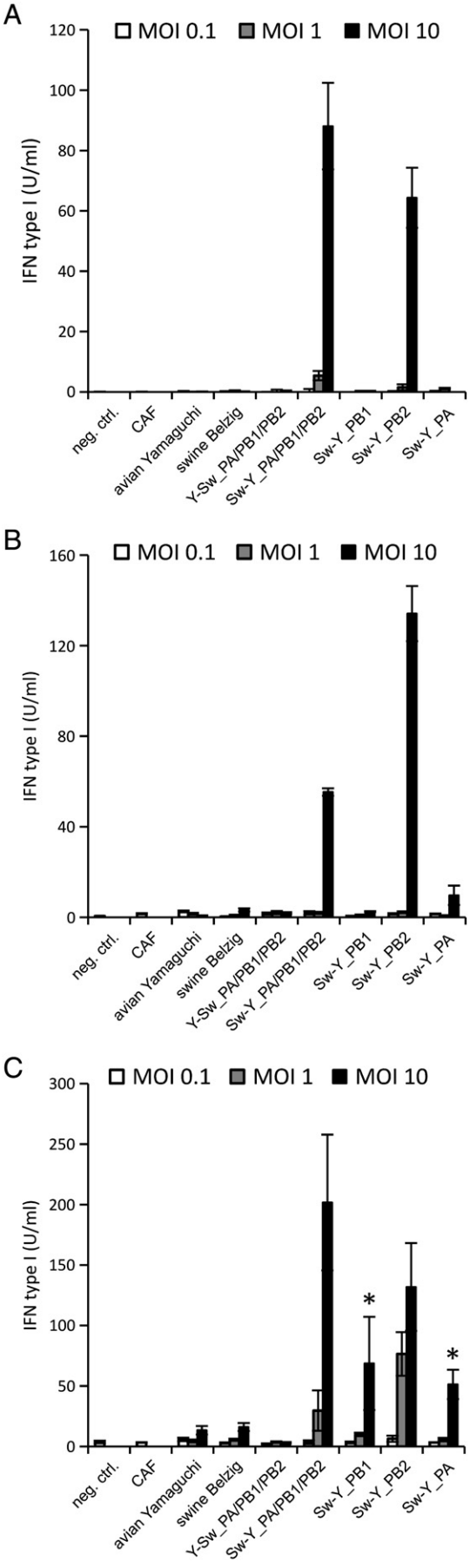
We also investigated the MHCII upregulation induced by single polymerase gene reassortants. Again, all viruses at an MOI of 1 and 10 IU/cell induced a statistically significant MHCII upregulation compared to CAF (Fig. 5B, $p<0.01$; calculated by *t* test). Only a tendency for enhanced effects was found with the swine H1N1 containing the avian PB2 polymerase (Fig. 5B).

Avian PB2 expression is associated with higher NF-κB activation of porcine DCs

Viral PB2 was reported to interfere with the mitochondrial antiviral signaling protein (MAVS) involved in NF-κB translocation to the nucleus (Graef et al., 2010; Iwai et al., 2010). Therefore, we investigated nuclear NF-κB in DCs 3 h p.i. with IAV. We observed the highest translocation of NF-κB to the nucleus after infection with swine viruses carrying the avian PB2. Statistical analysis showed significant differences to the control CAF with the Sw-Y_PA/PB1/PB2 and Sw-Y_PB2 but not with the original swine IAV or the Sw-Y_PB1 of Sw-Y_PB2 virus (Fig. 6A; $p<0.05$, calculated by *t* test). These results were confirmed by NF-κB binding to its DNA consensus sequence by ELISA. Only in nuclear from Sw-Y_PA/PB1/PB2 and Sw-Y_PB2 infected DC a binding activity was observed (Fig. 6B). No NF-κB binding was observed with cytosolic extracts (data not shown). This coincided with our data obtained for the IFN type I secretion.

Discussion

To cause an infectious disease in a particular host, IAV has to adapt to the receptor specificity and replication machinery of its main target cells in the epithelium of the respiratory or digestive tract. Nevertheless, thereafter the virus has to overcome the host immune defense. Based on the central role of DC in antiviral immunity, we focussed on this interaction in the present study. While all viruses studied efficiently infected the DC and had a potent impact on DC activity when measured in terms of MHCII up-regulation, only reassortants containing the avian PB2 gene were able to induce an early IFN type I



response in GM-CSF-induced bone marrow-derived DC as a model for conventional DC.

Although receptor recognition on the host cell surface by HA is the first crucial step for the virus to establish a successful infection, we did not observe differences in infectivity between viruses carrying the HA of the avian H5N1 compared to the swine H1N1. The DC were highly susceptible to infection and expressed high levels of NP, independent of the HA expressed by the viruses. This was somewhat surprising considering the important role played by the HA for infection of human endothelial cells and porcine plasmacytoid DC (Bel et al., 2011; Ocana-Macchi et al., 2009). The next step during infection is the release of the viral RNA into the cytoplasma. For IAV, this requires HA fusion with the endosomal membrane after HA cleavage. Highly pathogenic avian IAV have a polybasic amino acid (AA) cleavage site, which allows cleavage by ubiquitously expressed proteinases and facilitates systemic viral spread inside the host. All other IAV possess monobasic AA cleavage sites, restricting their replication in mammals mainly to the respiratory tract (Horimoto and Kawaoka, 2005). Our data indicate that in DC cultures viruses with polybasic cleavage site can multiply more efficiently resulting in measurable titers, although at low level. Interestingly, this did neither have an impact on DC activation in terms of MHCII expression nor on IFN type secretion. Nevertheless, the sharp increase in NP^+ between 5 and 20 h p.i. found with all viruses after infection with an M.O.I. of 0.1 IU/cell, indicates that also viruses with monobasic cleavage site can terminate a full replication cycle and infect neighboring cells in the culture, although no increase of virus titres in the supernatants were found.

Antiviral responses should be initiated in particular when viral replication starts, and for IAV this is mainly counteracted by NS1, which blocks the antiviral machinery of the host cell at various points and thereby enhances virus replication (Hale et al., 2008). In our DC culture model, we were unable to find differences in the avian and porcine NS1 to prevent DC activation (data not shown). Only recently, the importance of other viral proteins, such as PB1 and PB2, influencing the antiviral response became evident (Graef et al., 2010; Iwai et al., 2010; Le Goffic et al., 2010). For porcine DC, we identified PB2 as important viral element controlling IFN type I. While all viruses had a comparable ability to infect DC, to initiate replication and to activate the cells in terms of MHCII induction, only those viruses expressing the PB2 derived from the H5N1 virus were unable to prevent IFN type I induction. Altogether, this led us to the conclusion that in porcine DC the IFN antagonistic activity of avian PB2 is weaker compared to a PB2 from a porcine H1N1. This related to the observation that PB2 of human seasonal IAV localize not only to the nucleus, but also to the mitochondria, not observed with PB2 of avian IAV (Graef et al., 2010). In fact, PB2 is known to be able to interact with MAVS (Graef et al., 2010; Iwai et al., 2010), an essential protein in the signaling cascade of RNA helicases known to be important in sensing IAV in conventional DC (Summerfield and McCullough, 2009). MAVS signaling results in NF-κB activation and its subsequent translocation to the nucleus. Our observation that viruses expressing avian PB2 induced more NF-κB translocation compared to those expressing the porcine PB2 would indicate that differential interaction of the avian and porcine PB2 with the porcine MAVS in DC could possibly explain our observations. However, this needs to be clarified using biochemical techniques.

Graef et al. also showed that a single AA mutation at position 9 in the PB2 protein is sufficient to change its localization. PB2 encoding for an aspartic acid at position 9 did not localize to the mitochondria (Graef et al., 2010). However, both PB2 tested in our experiments contain an aspartic acid at position 9, indicating that this AA change cannot explain the differences in IFN induction in our DC model The two sequences of

Fig. 4. Avian PB2 suffices to change IFN inducing properties of porcine IAV in DCs. GM-CSF DCs were infected at three different MOIs (0.1, 1, 10 IU/cell). 5 h p.i. supernatants were harvested and tested for the presence of IFN type I by bioassay. Data for three experiments done in triplicates cultures are shown (A–C). *IFN type I induction by Sw-Y_PB1 and Sw-Y_PA differed significantly from the values obtained with Sw-Y_PA/PB1/PB2 ($p<0.05$).

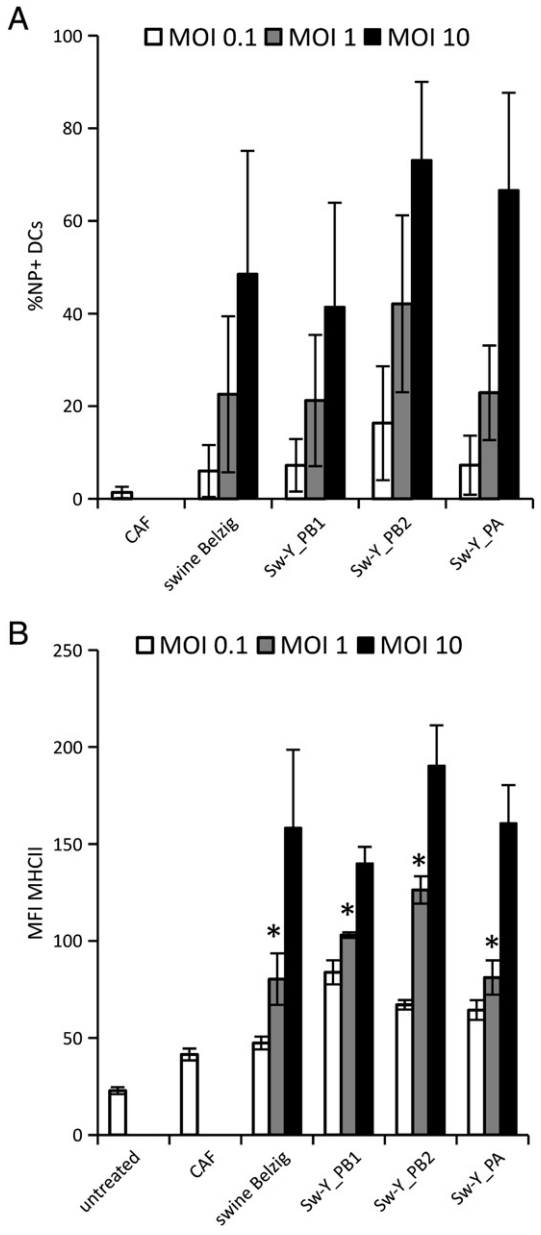


Fig. 5. NP and MHCII expression after infection of DC by single polymerase reassortants. GM-CSF derived DCs were infected at an MOI of 0.1, 1, 10 IU/cell and analyzed for NP at 5 h p.i. (A) and for MHCII expression at 24 h p.i. (B). In A and B, no statistical significant differences were found between the viruses employed when comparisons were made within one MOI. For an MOI of 1 and 10 IU/cell all viruses induced significantly higher expression of MHCII when compared to the CAF control (*, $p<0.01$). Data for a representative experiment out of two done in triplicates cultures are shown.

the PB2 used by us differ in 17 AA (see Table 1). They are identical at position 627 (glutamic acid), which is an important adaptive mutation site for efficient replication in pigs and humans, at least with certain viruses (Hatta et al., 2007; Manzoor et al., 2009; Massin et al., 2001). A glutamic acid at position 627 is not uncommon in swine IAV (Bussey et al., 2010), and it has been recently demonstrated that the glutamic acid to lysine mutation at this position can have a strong impact in mice but not in pigs (Ma et al., 2011). We therefore think that at least this could explain why reassortants with the avian PB2 possessed similar growth characteristics in porcine cells as those with the swine PB2. On the other hand, the Belzig and Yamaguchi strains differ at Position 701 (Gabriel et al., 2005). The swine virus PB2 gene encodes for an asparagine and the avian for an aspartic acid. This D701N mutation has been shown to increase association rates of PB2 to different importin isoforms in mammalian cells (Gabriel et al., 2011) which is important for successful replication (Boivin and Hart, 2011; Gabriel et al., 2008). However, neither in DC nor in the porcine PK-15 epithelial cell line, any differences in replication efficiency between all the viruses tested were observed (data not shown for the PK-15 cells).

It could have been expected that the Yamaguchi virus would also induce IFN type I in porcine DC, considering the influence of its PB2 on such responses but this was not observed. A possible explanation could be that the NS1 of the H5N1 is a more potent IFN antagonist and therefore IFN type I responses are only induced when the avian PB2 is combined with a porcine NS1. It is known that NS1 represents a virulence factor with a multitude of mechanisms and functions in regulating replication and innate immunity, and these do vary with various IAV (Hale et al., 2008). In this context it is interesting to note that the NS1 protein of the Yamaguchi virus was described to be a more potent IFN antagonist than that of a low pathogenic H5N1 virus, at least in chicken macrophages (Liniger et al., 2012).

Due to its function to inhibit expression of IFN type I in DC, PB2 could be one of the key factors for successful transmission of a new avian IAV into the swine and human population. We speculate that a reassortant virus with an avian PB2 which is inefficient in inhibiting IFN secretion would induce stronger innate and adaptive immune responses. This may restrict its replication resulting in mild clinical manifestation. On the other hand, inefficient PB2 could lead to enhanced inflammatory responses and more severe respiratory symptoms. This may depend on the immune status of the host.

Taken together, our data indicate that the PB2 of avian strains has to adapt to the porcine or human host in order to circumvent IFN expression.

Material and methods

Cells

Madin-Darby canine kidney (MDCK) cells were propagated in MEM (Invitrogen Basel, CH) supplemented with 10% fetal bovine serum (FBS; Biowest), non-essential AAs (NeAA; Invitrogen) and 1 mM sodium pyruvate (NaPyr; Invitrogen). Human embryonic kidney (HEK) 293T cells were propagated in DMEM GlutaMax without phenol red (Invitrogen) supplemented with 10% FBS. The swine kidney cell line SK-6 (kindly provided by Dr. M. Penseart, Faculty of Veterinary Medicine, Ghent, Belgium) was cultured in MEM supplemented with 7% horse serum (SVA, Hatunaholm, Sweden). DF-1 cells (chicken fibroblast cell line, ATCC) were cultured in DMEM (Invitrogen, Basel, CH) supplemented with 10% FBS and 1× GlutaMax (Invitrogen).

Porcine bone-marrow hematopoietic stem cells (BMHC) were isolated from a sternum and cultured in RPMI medium (Invitrogen) supplemented with 1× GlutaMax (Invitrogen), 1× Penicillin/Streptavidin (Invitrogen), 10% FBS and in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF) as previously described (Carrasco et al., 2001) for two weeks before infections.

Viruses

The following viruses were used A/chicken/Yamaguchi/7/04 (H5N1) and A/swine/Belzig/2/01 (H1N1). All viruses were propagated in 10-day-old embryonated, specific-pathogen-free chicken eggs. We followed the protocol of the *WHO Manual on Animal Influenza Diagnosis and Surveillance* with some minor changes. Instead of 0.1 ml we inoculated 0.2 ml of different dilutions of virus. The inoculated eggs were incubated for 24 h (HPAIV) or 48 h (other influenza viruses).

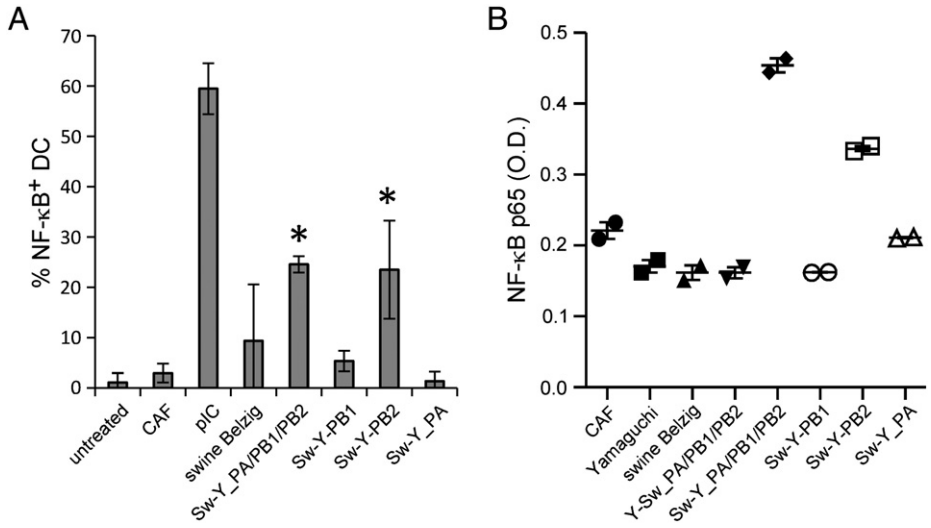


Fig. 6. NF-κB translocation after IAV infection of DC. GM-CSF derived DCs were infected at an MOI of 1 IU/cell, harvested 3 h p.i. for isolation of nuclei and NF-κB quantification. In A, the mean of percentage of NF-κB$^+$ nuclei is shown for one experiment done with triplicate cultures. Only Y-Sw_PA/PB1/PB2, Sw-Y_PA/PB1/PB2 and Sw-_PB2 induced significantly higher percentage of NF-κB$^+$ nuclei compared to CAF (*). In B, nuclear extracts from an independent experiment were extracted, and the binding of NF-κB to an oligonucleotide containing the NF-κB consensus binding site was quantified by ELISA (TransAM™, Activemotif). Duplicates of the optical density (O.D.) are shown.

Reassortants generated by reverse genetics

The following viruses were obtained by reverse genetics (RG) as previously described (Ocana-Macchi et al., 2009). These included a Yamaguchi H5N1 virus containing the polymerase genes PA, PB1 and PB2 of the swine H1N1 virus (Y-Sw_PA/PB1/PB2), a swine H1N1 virus containing all Yamaguchi polymerase segments (Sw-Y_PA/PB1/PB2), a swine H1N1 virus containing the Yamaguchi PA (Sw-Y_PA); a swine H1N1 virus containing Yamaguchi PB1 (Sw-Y_PB1) and a swine H1N1 virus containing Yamaguchi PB2 segment (Sw-Y_PB2).

Virus titrations

Calculations of multiplicities of infection (MOI) were based on infectious units (IU) on DF-1 cells, determined using a protocol adapted from (Matrosovich et al., 2006). 6 h after titration, cells were washed with PBS, and then fixed with 4% paraformaldehyde (Sigma) solution in PBS for 15 min at room temperature (RT), washed and permeabilized with 100 µl/well of PBS containing 0.5% Triton-X-100 and 20 mM glycine. Viral nucleoprotein (NP) detection was performed by incubating cells for 1 h with monoclonal antibody HB65 (ATCC) diluted in PBS supplemented with 10% horse serum (HS, Häst) and 0.05% Tween80. Cells were then washed 5 times with PBS-0.05% Tween80, followed by 1 h incubation with peroxidase-labeled rabbit anti-mouse antibodies (DAKO, Zug Switzerland) diluted 1:1000 in PBS supplemented with 10% HS and 0.05% Tween80. Cells were washed again 5 times with PBS-0.05% Tween80 and then incubated for 15 min with TrueBlue substrate (KPL, MD, U.S.A.). Plates were washed with tap water and then dried. Single infected cells were counted under the microscope to determine IU/ml.

Viral titres in cell culture supernatants were determined as $TCID_{50}$/ml. To this end, supernatants were titrated on MDCK in 96-well plates in the presence of 1 µg/ml TPCK trypsin (Sigma). 72 h p.i. cells were washed and stained with crystal violet. $TCID_{50}$/ml was calculated according to the Reed-Muench formula (*WHO manual on Animal Influenza Diagnosis and Surveillance*).

Infection of GM-CSF derived DCs

The DCs were distributed in 15 ml polypropylene tubes at a concentration of 10^6 cells/ml in RPMI supplemented with 0.125% BSA (Intergen, NY), 1 mM HEPES (Invitrogen), and 1× GlutaMax (Invitrogen), and infected with an MOI of 0.1, 1 and 10 IU/cell. As negative control for infection, the DCs were incubated with CAF. After 1 h incubation at 4 °C cells were washed, and cultured in 24-well plates at a concentration of 10^6 cells/ml, 39 °C, 5% CO_2. As positive control for activation, we added 10 µg/ml polyinosine-polycytidylic acid (polyIC, Sigma).

Flow cytometry

For nuclear protein (NP) detection, cells were harvested with PBS-A (Invitrogen), 0.94 mM EDTA, and then washed with Cell Wash (Becton Dickinson). Cells were fixed and permeabilized with Fix & Perm cell permeabilization kit (An der Grub, Austria) according to the manufactures instructions, and labeled using anti-NP clone HB65 (ATCC) as described (Ocana-Macchi et al., 2009). For MHCII staining we employed monoclonal antibody MSA3 (hybridoma kindly provided by Dr. A. Saalmüller, Veterinary University, Vienna, Austria) for 20 min on ice, washed with cell wash and then incubated with RPE-conjugated goat-anti mouse IgG2a antibody for 20 min on ice. After washing, cells were fixed with 4% paraformaldehyde for 15 min, washed again and resuspended in 200 µl Cell Wash for acquisition.

NF-κB activation

To measure NF-κB translocation, GM-CSF DCs were infected at an MOI of 1 IU/cell for 1 h on ice, washed, resuspended in growth medium and incubated at 39 °C, 5% CO_2 for 3 h. Then the DC were harvested, washed with PBS containing 1% BSA, 0.05% NaN_3 (washing

Table 1
Differences in AA sequence of swine Belzig and avian Yamaguchi PB2.

Position	61	64	67	107	108	251	295	339	442	446	483	495	588	613	649	667	701
Swine Belzig	R	M	I	N	T	K	I	K	S	V	T	A	N	A	I	I	N
Avian Yamaguchi	K	I	V	S	A	R	V	T	A	F	M	V	A	V	V	V	D

buffer) and nuclei released with a PIPES based lysis buffer (10 mM PIPES, 0.1 M NaCl, 2 mM $MgCl_2$, 0.1% triton X100, pH 6.9) for 30 min on ice. After lysis 2 ml washing buffer were added to the nuclei and then centrifuged for 5 min at 350 *g* and 4 °C. This washing step was repeated once and nuclei were then incubated with anti-NF-κB p65 (Santa Cruz Biotechnology) for 30 min on ice, washed twice with washing buffer and stained with RPE-conjugated goat-anti mouse IgG1 antibody (Southern Biotech) for 30 min on ice. After a final washing step, nuclei were resuspended in 200 µl washing buffer. All data were acquired with a FACSCalibur (Becton Dickinson) and analyzed with FlowJo software (Treestar Inc. Ashland, OR). The DNA binding capacity of NF-κB to the consensus binding motif also quantified by ELISA using plates coated with the corresponding oligonucleotide following the manufactures instruction (Activemotif, TransAM™ p65 assay kit). Cytoplasmic extract were prepared as described (de Los Santos et al., 2007) by lysing cellular membranes in a low salt buffer (10 mM HEPES pH7.9, 10 mM NaCl, 3 mM MgCl2 0.5% NP-40) for 20 min at 4 °C, followed by centrifugation of the remaining nuclei at 3200 *g* for 10 min. After washing the pellet with low salt buffer (3200 *g*, 10 min 4 °C), it was resuspended in high salt buffer (20 mM HEPES, 25% glycerol, 0.42 M KCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol) and incubated for 20 min at 4 °C. The nuclear lysates were then clarified by a final centrifugation step (3200 *g*, 10 min, 4 °C).

IFN type I bioassay

To determine IFN type I bioactivity, SK6-MxLuc cells were employed. This is a porcine kidney cell line stably expressing firefly luciferase under the control of the murine Mx1 promoter (Ocana-Macchi et al., 2009). SK6-MxLuc cells were seeded in 96-well plates at $2 \cdot 10^4$ cells/well. The next day supernatants of infected GM-CSF derived DCs were inactivated by UV treatment to avoid direct stimulation of the luciferase transcription by the virus. To this end, 50 µl supernatant were distributed in one well of a 96-well plate which was irradiated with 9999 mJ UV light. The standard was treated the same way. SK6-MxLuc cells were washed and 50 µl of fresh medium were added together with 50 µl of supernatants. 18 h later supernatants were removed, the cells lysed with 200 µl cell culture lysis reagent provided by Promega luciferase assay kit (Promega, WI, USA), and luciferase activity was measured with a luminometer.

Reverse transcriptase RT-PCR

GM-CSF DCs were infected at an MOI of 10 IU/cell as described above. 5 h p.i. cells were harvested. Total RNA was isolated using TRIZOL (Invitrogen) according to manufacturer's protocol and treated with RNase-free DNase I (Ambion, Huntington, UK). The real-time reverse transcription PCR (real-time RT PCR) was performed with SuperScript™ III Platinum® one-step qRT-PCR System (Invitrogen) using the ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA, USA). The relative expression of each mRNA was calculated by the ΔCt method, and the amount of IFN-α and-β mRNA relative to 18S mRNA was expressed as 2^{-Ct} (Livak and Schmittgen, 2001). The following primers and probes were used: 18S forward primer 5′-CGCCGCTAGAGGTGAAATTC-3′; 18S reverse primer 5′-GGCAAATGCTTTCGCTCTG-3′; 18S probe 5′-TGGACCGGCGCAAGACGGA-3′; IFN-β forward primer 5′-GGCTGGAATGAAACCGTCAT-3′; IFN-β reverse primer 5′-TCCAGGATTGTCTCCAGGTCA-3′; IFN-β probe 5′-CCTTGTGGAACTTGATGGGCAGATGG-3′; IFN-α forward primer 5′ TGTGCTGAAGAGCTGGAAGGT-3′; IFN-α reverse primer 5′-CAGGTCCAGAAGGCTCAAGC-3′; IFN-α probe 5′-TGGCTCTGGTGCATGAGATGCTCC-3′; M1 forward primer 5′-AGATGAGYCTTCTAACCGA-3′; M1 reverse primer 5′-GCAAAGACATCTTCAAGTYTC-3′; M1 probe 5′-TCAGGCCCCCTCAAAGCCGA-3′.

After 1 h of incubation at 4 °C, cells were washed five times with PBS (4 °C) and lysed with 400 µl lysis buffer containing a defined amount of enhanced green fluorescent protein (EGFP) RNA (Machete-Nagel kit nucleospin multi 96-virus). The RNA extraction was done by a Tecan Freedom EVO Roboter, and M1 and EGFP RNA were amplified by real-time reverse transcriptase PCR. Primers and probe with the following sequences were used: 5_-TCAGGCCCCCTCAAAGC CGA-3_ (probe), 5_-AGATGAGYCTTCTAACCGA-3_ (forward primer), and 5_-GCAAAGACATCTTCAAGTYTC-3_ (reverse primer). RNA was amplified using the following protocol: 30 min at 48 °C, 10 min at 95 °C, 15 s at 95 °C, 60 s at 54 °C, and 60 s at 70 °C. The last three steps were repeated 45 times. Threshold cycle (*CT*) values were corrected to the amount of EGFP RNA. To compare the virus binding, the dilutions at which virus binding to the cells was not saturated and with *CT* values below 30 were chosen. The relative amount of viral RNA was calculated by the Δ*CT* method, and the amount of viral RNA relative to EGFP RNA was expressed as 2Δ*CT* (18).

Statistics

P values were calculated by an unpaired *t* test in a SigmaPlot. Error bars representing the standard deviations of at least three experiments or one representative experiment done in triplicates were calculated by Excel.

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